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Electrophoretic Protein Patterns and Enzyme Mobilities in Anaerobic Coryneforms

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The soluble protein patterns and electrophoretic mobilities of malate and succinate dehydrogenases and catalase have been examined in 25 strains of *Propionibacterium acnes*, *P. granulosum*, and *P. avidum*. A distinctive protein pattern for each species was found, and it was possible also to distinguish the serotypes within *P. acnes* and *P. avidum*. Strains of *P. acnes*, *P. granulosum*, and *P. avidum* could be differentiated by the mobilities of their malate dehydrogenases. Catalase activity was detected in the soluble fractions of all strains. Catalases from *P. acnes* and *P. avidum* strains had the same mobility, whereas that from *P. granulosum* was slightly slower. Under the conditions used, succinate dehydrogenase activity could be detected, but the patterns were not distinctive.

Examination of cytoplasmic protein patterns and the electrophoretic mobilities of specific enzymes have been found to be useful in the classification of several groups of microorganisms, e.g., lactobacilli (4), leptospira (5), and bacteroides (8). The present study reports the results of an investigation into the protein patterns and enzyme mobilities in a number of strains of the anaerobic coryneforms *Propionibacterium acnes*, *P. avidum*, and *P. granulosum*. These species were originally defined by deoxyribonucleic acid homology relationships and cell wall studies (3, 7), and it has been shown that *P. acnes* and *P. avidum* can each be further subdivided into two serotypes (2).

The soluble protein patterns and mobility of catalase were examined by electrophoresis in polyacrylamide gel slabs, and the mobilities of malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) were determined on cellulose acetate strips (Cellogel).

MATERIALS AND METHODS

Strains used. A list of strains examined for protein patterns and dehydrogenase activity is given in Table 1; some additional strains were examined for catalase only and have not been included in the table. The standard strains used were: *P. acnes* type I, VPI 3706; *P. acnes* type II, VPI 0162; *P. avidum* type I, VPI 0575; *P. avidum* type II, VPI 0589; and *P. granulosum*, VPI 0507. (For the origins of these strains see reference 2.)

All strains were maintained in anaerobic chopped-meat medium (6) and were verified as to species and serotype by immunodiffusion tests with standard antisera prepared against cell wall material from each of the type strains.

Culture growth and preparation of soluble fractions. Organisms were grown in peptone-yeast extract-glucose-Tween 80 medium (6) at 37°C. Two milliliters of a good growing 24-h culture was inoculated into each of two tubes containing 75 ml of this medium. After 24 h cells were harvested by centrifugation, washed three times in distilled water, suspended in cold 0.05 M phosphate buffer (pH 6.8), and disrupted with glass beads (0.11 mm) in a Braun disintegrator, using a ratio of 1 part glass beads to 1 part cell suspension. After filtration through a coarse fritted glass filter to remove beads followed by high-speed centrifugation (30,000 × *g*, 15 min), the protein contents of the soluble fractions were estimated by the method of Lowry et al. (10), and for polyacrylamide gel electrophoresis the protein contents were adjusted to a concentration of 1 mg of protein per ml. Fractions were stored at −15 to −18°C.

Polyacrylamide slab gel electrophoresis. Vertical anionic gel electrophoresis in gels (10 by 14 cm) using 0.025 M tris(hydroxymethyl)aminomethane glycine buffer (pH 8.3) was carried out with 7.5% polyacrylamide gel and 4% spacer by a method adapted from that of Laemmli (9), omitting sodium dodecyl sulfate and ethylenediaminetetraacetate. Approximately 30-μl portions of sample (=30 μg of protein) in 30% sucrose were loaded into the slots, and gels were electrophoresed at 200 V and 15 mA for 4 to 5 h at 4°C. Bromophenol blue was used as a tracking dye.

Cellulose acetate electrophoresis. Cellogel strips (2.5 by 17 cm; Kalex Scientific Co., Manhasset, N.Y.) were employed. Samples (10 μl) of soluble fraction from the bacterial strains were applied to the strips, and electrophoresis was carried out for 2 h at 4°C in 0.04 M sodium barbital buffer, pH 9.2, at 1.0 to 1.5 mA/strip and at a starting potential of 200 V.

Staining methods. (i) Soluble proteins. Gels were stained with 2% Coomassie brilliant blue in water-methanol-acetic acid (4.5:4.5:1) (1) for 1 to 2 h at 37°C, destained by agitation in the same solvent without dye, and stored in 7% acetic acid.

(ii) Dehydrogenase activity. Localization of MDH, SDH, and other dehydrogenase activities was

TABLE 1. *List of strains*

Type of organism	VPI strain no.	Other designations	Site of origin
P. acnes type I	0208	Prevot 2683	Blood culture
	3706		Human skin
	6572	Prevot 3239, *Corynebacterium parvum*	Inguinal gland puncture
	6630	Prevot 3594, *C. parvum*	Blood culture
	6642	Prevot 2706, *C. parvum*	Blood Culture
P. acnes type II	0162	Prevot 578, *C. anaerobium*	
	6626	Prevot 3879, *C. parvum*	Vulvo-vaginitis
	6632	Prevot 2355-A, *C. parvum*	Abscess of neck
	6639	Prevot 2484C	
	6641	Prevot 2738	
P. avidum type I	0575	CDC 71	Hip sinus
	4160	Prevot 2068, *C. acnes*	
	6451	CDC B7706	Pelvic abscess
	6452	CDC B7712	Buttock swab
	6581	Prevot 2782	Inguinal abscess
P. avidum type II	0179	ATCC 25577, Prevot 1689B, *C. avidum*	
	0589	CDC A518	Wound swab
	0670	CDC 6440A	Earlobe cyst
	4982-2	London Hospital, London, England	Normal skin
	6631	Prevot 3232D, *C. parvum*	Allergic rhinitis
P. granulosum	0507	ATCC 25564	Culture contaminant
	9556	Evans 73-710	Normal skin, forehead
	9559	Evans 74-150	Normal skin, forehead
	9566	Evans 74-134	Comedone
	9578	Evans 74–423	Acne comedone

carried out by an adaptation of the procedure of Wieme (12). The required substrate (1 M solution) was added to a mixture of nicotinamide adenine dinucleotide, phenazine methosulfoxalate, and iodonitrotetrazolium violet (proportions as in reference 12). After electrophoresis, the Cellogel strips or polyacrylamide gels were incubated at 37°C in the mixture until reddish-violet lines appeared, indicating the site of dehydrogenase activity. The reaction was then stopped by transferring the strip or gel to 7% acetic acid.

The distances moved on the strips by the leading and trailing edges of bands were measured, and the mean value was calculated. The R_f values were calculated with reference to a preparation from VPI 0162 (*P. acnes* type II), which was included in each run.

(iii) Catalase activity. For catalase assay, gels were incubated at 37°C in 3% H_2O_2 until bubbles appeared, then washed three times in distilled water and flooded with 2% potassium iodide in 2% acetic acid for 1 min in a modification of the method of Thorup et al. (11). Gels were then covered briefly with Whatman 3MM paper impregnated with 1% soluble starch. Areas of catalase activity were indicated by colorless regions in the gel.

RESULTS

Soluble protein patterns found in acrylamide gels. The main bands found in preparations from strains of *P. acnes*, *P. avidum*, and *P. granulosum* are shown in Fig. 1. The *P. granulosum* preparation shown in the photograph is from a different gel slab, but has been aligned with the others for comparison. All protein patterns are based on preparations from 24-h cultures, since the relative intensities of the bands may change with time. At 24 h, cultures of *P. avidum* are entering the stationary phase of growth, while cultures of *P. acnes* and *P. granulosum* are in the late log phase. The bands characteristic of species differences are as follows.

(i) *P. acnes*. The distinguishing features are rather narrow bands at 1.5 cm compared with the broader, rather faster bands in *P. avidum*, and a very distinct band at about 6.5 cm, which is not present in *P. avidum* strains.

P. acnes types I and II can be distinguished by the rather broader band at 1.5 cm, which is characteristic of type II, the presence of a double band at 5 cm in type I, and the differences in band pattern at 7 to 8.5 cm.

(ii) *P. avidum*. The main distinguishing features of *P. avidum* strains are the intense broad bands at about 2.0 cm, the intense double bands at about 5.0 and 5.5 cm (not present in *P. acnes*), and the well-marked bands at 8.5 to 10.00 cm.

P. avidum types I and II are distinguished by

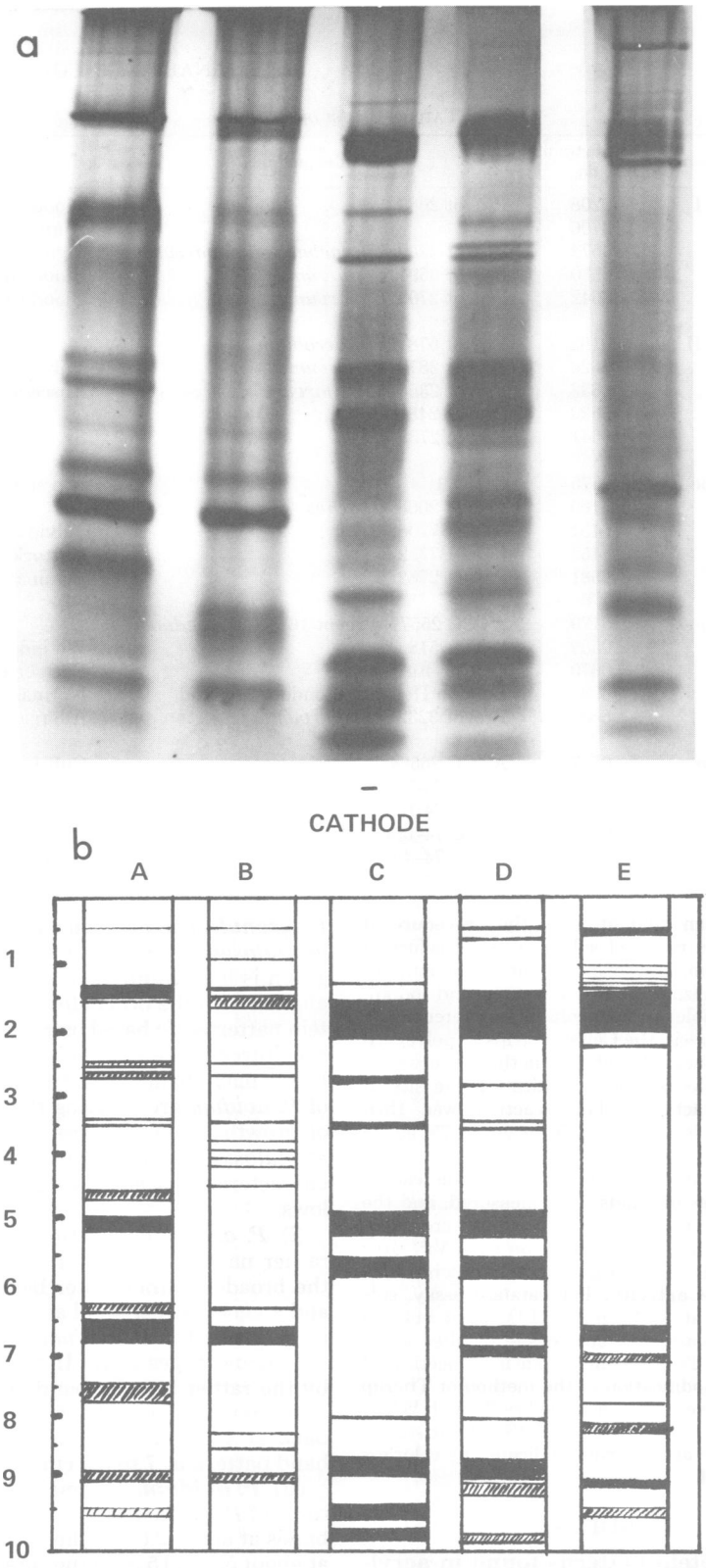

FIG. 1a & b.

the sharp double bands at 3.5 cm in type II compared with the single band in type I, by the double band in type II at approximately 7.0 cm, and by the differences in the bands at 8.5 to 10.00 cm. In the latter zone, the three bands in *P. avidum* type I are equally spaced and of about equal intensity, whereas in type II, the slowest band (9 cm) is the strongest of the three.

(iii) *P. granulosum*. The main features are the intense slow-moving band at about 0.75 cm, the multiple narrow bands at 1 to 2 cm, and the long featureless area between 2 and 6.5 cm.

Enzyme activities. (i) MDH. MDH was demonstrated in all cultures both by polyacrylamide gel and cellulose acetate electrophoresis (Fig. 2).

The MDH enzymes in the three species had different mobilities and, in the case of *P. acnes*, the enzymes from types I and II also differed. With both electrophoretic methods, MDH from *P. acnes* had the lowest mobility, with the type II enzymes moving faster than those from type I strains. MDH from *P. avidum* strains ran faster than those of *P. acnes* of either type, but the enzymes from *P. avidum* I and *P. avidum* II could not be distinguished.

The bands of enzyme activity in preparations from *P. granulosum* strains generally ran a greater distance than those of the other species, but were of a somewhat different character, since the zones were more than twice as wide and were diffuse in appearance. Fresh preparations usually showed three distinct but closely associated bands, but as preparations aged, a single diffuse band often appeared instead of the three separate lines.

The preparations from *P. avidum* and *P. acnes* generally gave strong, well-defined single bands of activity. The enzymes appeared stable, and activity could still easily be detected after storage and repeated freeze-thawing. On several occasions, fresh preparations of *P. acnes* type II strains showed an additional band of enzyme activity of greater mobility than the normal line (R_f about 1.16). This was not seen in stored preparations and was assumed to be a labile isoenzyme.

(ii) SDH. SDH activity was found to be present consistently in *P. acnes*, *P. avidum*, and *P. granulosum* strains, but at a much lower level than MDH activity. On Cellogel strips, *P. acnes* and *P. avidum* soluble fractions gave SDH lines of similar mobility, whereas *P. granulosum* strains showed greater variability in R_f values. All three species often produced some streaking due to diffuse SDH activity between the origin and the principal SDH band.

When polyacrylamide gel was employed, areas of SDH activity were retarded by the 7.5% gel and largely remained at the base of the spacer gel. The streaking on cellulose acetate and the retardation on polyacrylamide gel suggest that the enzyme is aggregated and possibly membrane bound. Further evidence of binding was found in the fact that streaking was not observed when electrophoresis was performed in the presence of sodium dodecyl sulfate.

The enzyme was much more labile than MDH. Activity was frequently lost during preparation of the soluble fractions and rapidly disappeared if they were subjected to freeze-thawing.

(iii) Catalase activity. Catalase activity was detected in strains of all three *Propionibacterium* species examined (a total of 35 strains) when soluble fractions were run on polyacrylamide gel. Areas of activity from strains of *P. acnes* and *P. avidum* ran the same distance (approximately 5 cm in 5 h), whereas areas of *P. granulosum* catalase activity moved a slightly shorter distance.

DISCUSSION

The protein patterns obtained after Coomassie brilliant blue staining of polyacrylamide gel electrophoresis runs have proved to be highly distinctive and reproducible. A minimum of five strains of each type or species have been tested, and the characteristic bands have always been present. In the case of *P. acnes* and *P. avidum*, not only can the species be separated, but the two serotypes can be distinguished. Furthermore, during the course of the investigation, the protein banding patterns have enabled us to detect the fact that certain cultures were contaminated, since two strains supposed to be *P. granulosum* were found to give protein patterns corresponding to *P. acnes* serotype II. The contamination was confirmed by serological examination of the two cultures.

The soluble protein patterns on polyacrylamide gel stained with Coomassie brilliant blue indicate an overall similarity between *P. acnes* and *P. avidum* both in number and location of bands, whereas the protein pattern of *P. gran-*

FIG. 1. *(a) Photograph and (b) line drawing of soluble protein patterns in acrylamide gels found in representative strains of P. acnes, P. avidum, and P. granulosum. A, P. acnes serotype I, strain 3706; B, P. acnes serotype II, strain 0162; C, P. avidum serotype I, strain 0575; D, P. avidum serotype II, strain 0589; E, P. granulosum, strain 0507. Anode is at bottom.*

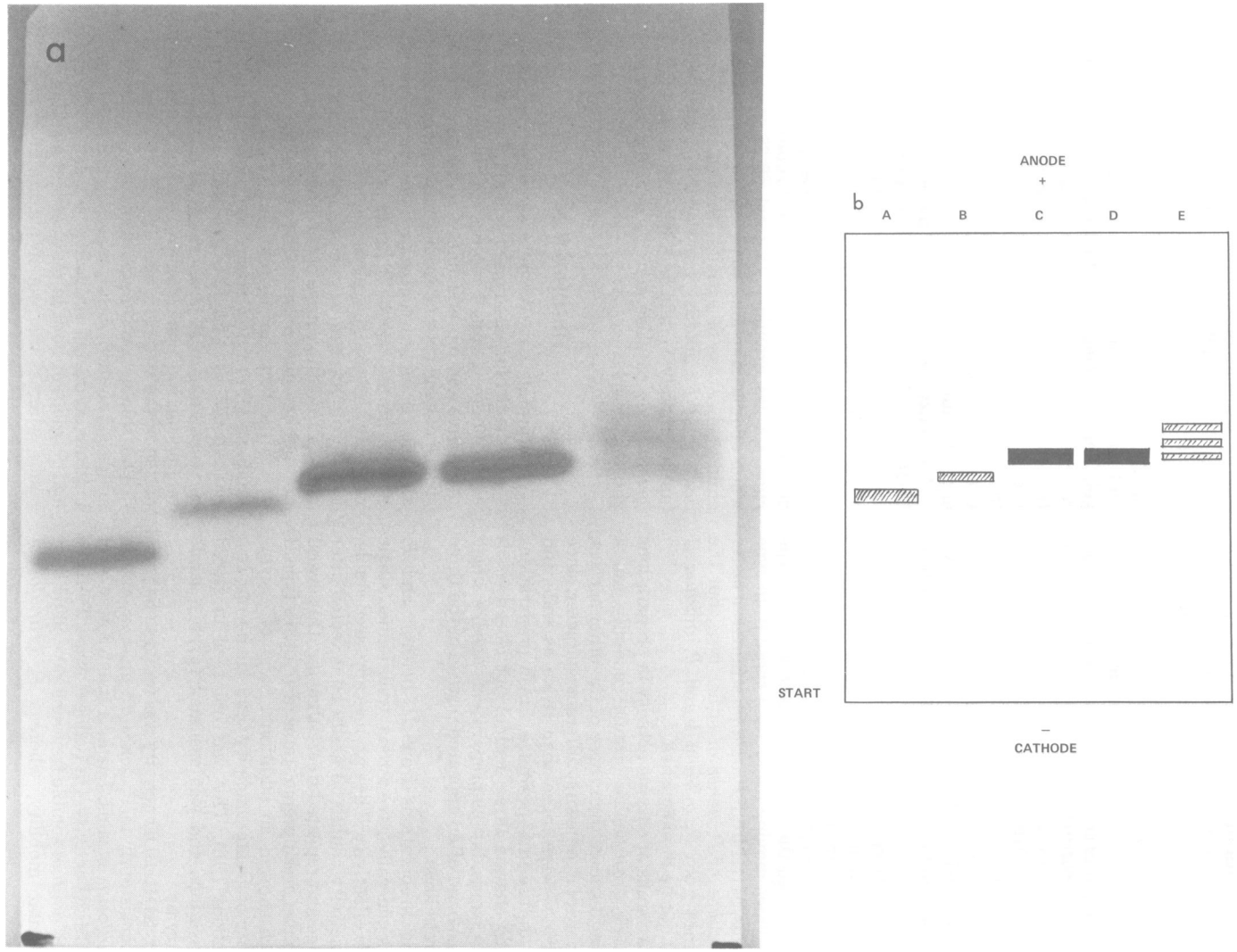

FIG. 2. *(a) Photograph and (b) line drawing of MDH activity in representative strains of P. acnes, P. avidum, and P. granulosum (strains A, B, C, D, and E as in Fig. 1). Anode is at top.*

ulosum is made up of fewer bands, only one of which corresponds to any band in *P. acnes* type I and II. The protein patterns of the three propionibacteria therefore confirm the results of earlier deoxyribonucleic acid homology and immunodiffusion studies (2, 7) which indicated that *P. acnes* and *P. avidum* are more closely related to one another than either is to *P. granulosum*.

The bands of MDH and catalase activity in *P. acnes* and *P. avidum* also bear greater resemblance to one another than to those in *P. granulosum*. The dense, single bands of MDH activity of *P. acnes* and *P. avidum* fractions, although differing in mobility, are readily distinguishable from the multiple or diffuse *P. granulosum* bands.

On the whole, R_f data of MDH activity were reproducible, and the same ratio of R_f values between species emerged repeatedly. The least consistent data came from *P. granulosum*, where variability may be related to changes in band character from multiple lines to a single diffuse band which depended to some extent on the age of the preparation.

It is apparent from the data that two dehydrogenases with different substrate specificities have been detected in these studies and not one enzyme with dual specificity. The SDH activity proved to be much less stable to the process of cell disruption and to storage, with enzyme activity no longer detectable after a week at −15°C. MDH activity, in contrast, maintained full activity after many weeks of storage. Although R_f values for SDH and MDH were occasionally similar, in the majority of cases, the values were different for a given strain, indicating the presence of two enzymes. There was less difference in the mobilities of SDH enzymes from the different species than was the case with MDH activity. With SDH, the R_f values varied from 0.92 to 1.05 as compared with the range of 0.85 to 1.13 for MDH.

The differences in behavior exhibited by the two enzymes when assayed for activity on polyacrylamide gels are further evidence that they are indeed different; SDH activity largely failed to migrate past the spacer gel, indicating aggregation of the enzyme into a high-molecular-weight polymer, whereas bands of MDH activity moved through the running gel at rates comparable to those on Cellogel strips. This behavior suggests that the different mobilities of enzymes with MDH activity are more likely to be due to differences in charge rather than size.

Both SDH and MDH are intermediates in the propionic acid fermentation pathway of classical propionibacteria, and the presence of enzymes associated with these intermediates now found in anaerobic coryneforms is preliminary evidence that the same pathway exists in this latter group also. The other two possible dehydrogenases in this pathway, lactate dehydrogenase and pyruvate dehydrogenase, were occasionally detected, but appeared very unstable under the conditions used and were not studied further. Failure to detect the latter two enzymes regularly does not appear to be related to interference by oxygen (13), since the reactions were not improved by using the detection system in a nitrogen atmosphere.

In assessing the general significance of the electrophoretic results, it may be useful to compare them with the results of immunodiffusion tests. In general, the two sets of results are entirely comparable, and there have been no major discrepancies. Technically speaking, immunodiffusion tests are simpler to perform, once suitable sera have been obtained. A further advantage of immunodiffusion tests is that the patterns are not dependent on the age of the culture, whereas when comparisons of electrophoretic patterns are to be made, it is probably important to use cultures at the same stage of the growth cycle.

However, these investigations were not primarily undertaken to develop a new identification procedure, but to see whether electrophoretic protein and enzyme patterns could be used as additional criteria in separating the species *P. acnes*, *P. avidum*, and *P. granulosum* and their serotypes. It seems clear from the results that the electrophoretic patterns are distinctive for each species and for the serotypes also. As far as the identification of unknown strains is concerned, we suggest that the method be used in parallel with the immunodiffusion test in doubtful cases. For example, we have encountered a strain identified as *P. avidum* by biochemical tests which did not react with the standard antiserum. However, in electrophoretic analysis, the protein pattern was typical of a *P. avidum* type I.

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